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## Cholera Toxin and Cholera Toxin B Subunit Induce IgA Switching Through the Action of TGF- $\beta$ 1<sup>1</sup>

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### ▶ Abstract

Cholera toxin (CT) and its B subunit (CTB) are potent immunogens and adjuvants that, either alone or linked to protein Ags, can stimulate mucosal immune responses, modulate the induction of oral tolerance, and stimulate IgA isotype switching. The present studies addressed the mechanisms by which CT and CTB promote IgA switching. CT and rCTB, in the presence of IL-2, significantly increased IgA isotype switching at the clonal level in populations of purified and LPS-activated murine surface IgA<sup>+</sup> spleen B cells, as determined by ELISA, enzyme linked immunospot assays, and limiting dilution analysis. The IgA stimulatory effects of CT and CTB were independent of the A subunit of CT. CTB and CT did not increase the secretory rate of IgA-producing cells or the clonal burst size of IgA clones, and did inhibit B cell growth. Because TGF- $\beta$ 1 also inhibits B cell growth and promotes IgA switching, further studies tested whether the activity of CTB and CT on IgA isotype switching was mediated through TGF- $\beta$ 1. Anti-TGF- $\beta$  Ab and soluble TGF- $\beta$ 1 type IIR inhibited CTB- and CT-stimulated IgA isotype switching. Furthermore, increased TGF- $\beta$ 1 mRNA levels and bioactive TGF- $\beta$ 1, within a range shown to induce IgA isotype switching, were detected in cultures of surface IgA<sup>+</sup> B cells stimulated with CT or CTB and IL-2. These data indicate that CTB- and CT-stimulated IgA isotype switching are mediated through TGF- $\beta$ 1. The finding that CTB up-regulates TGF- $\beta$ 1 activity has important implications for understanding the mechanisms by which CTB promotes both IgA mucosal immunity and oral tolerance.

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## Introduction

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Ig heavy chain constant region isotype switching results in the generation of Igs that can mediate a broad spectrum of effector functions that are optimally suited for the microenvironment in which they encounter Ag. This is particularly the case for IgA, which is the predominant Ig produced at mucosal surfaces. IgA plays a major role in Ag binding and mucosal defense, particularly against extramucosal intraluminal pathogens. Cytokines are essential in the regulation of IgA isotype switching and IgA B cell differentiation. Thus, TGF- $\beta$ 1 promotes heavy chain switch recombination to IgA in murine and human B cells (1, 2, 3, 4), whereas IL-2, IL-4, IL-5, IL-6, and IL-10 have been shown to a variable extent to have an important role in murine and human IgA B cell differentiation (3, 5, 6, 7, 8, 9, 10).

Cholera toxin (CT)<sup>3</sup>, an 86-kDa enterotoxin produced by *Vibrio cholerae*, consists of five B subunits of CT (CTB) that are arranged as a pentamer and are covalently linked to a single 33-kDa A subunit of CT (CTA) (11, 12). CTB can bind to G<sub>M1</sub> gangliosides on the surface of intestinal epithelial cells, lymphocytes, and other cells, after which CTA can enter the cell (13, 14). In the case of intestinal epithelial cells, this induces ADP ribosylation of a G<sub>s</sub> regulatory protein, which results in elevated levels of intracellular cAMP, increased Cl<sup>-</sup> secretion, and diarrhea (14), the hallmark of clinical cholera infection. CT is also a potent adjuvant. Thus, protein Ags, which themselves are not immunogenic by the enteric route, are rendered immunogenic when coadministered with CT (15, 16). In the intestine, the adjuvant effect of CT on mucosal immune responses results in marked up-regulation of local IgA responses and appears to be dependent on CD4 T cells, especially those belonging to the Th2 subset that produces IL-4 and IL-5 (17, 18). Moreover, CT is a potent mucosal immunogen and stimulates mucosal anti-CT IgA responses (19). In addition, CT in combination with IL-4 has been shown to increase the IgG1 response of LPS-activated spleen B cells in vitro (20).

CTB alone can also modulate mucosal immune responses. Thus, enteric administration of CTB, together with a protein Ag, increased murine Ag-specific mucosal IgA responses (21). In addition, oral or intranasal administration of CTB as a carrier conjugated to a protein resulted in the induction of local Ag-specific secretory IgA responses (22, 23). Moreover, enterically or intranasally administered proteins that were coupled to CTB were more effective than the proteins alone at inducing oral tolerance in mice (24, 25).

Several reports suggest CT and CTB can increase IgA isotype switching. Thus, CT and CTB increased the number of IgA-producing cells among populations of LPS-activated Peyer's patch B cells (26), CT increased the frequency of IgA-secreting cells in cultures of CH12.LX (surface IgM<sup>+</sup> (sIgM<sup>+</sup>)) murine B lymphoma cells (26, 27), and intraduodenal administration of CT increased the frequency of IgA precursor cells in Peyer's patches (28). In the studies herein, we report that CT and CTB increase IgA isotype switching at the clonal level, independent of CTA. Moreover, CTB-stimulated IgA isotype switching is mediated through TGF- $\beta$ 1 and requires IL-2 as a cofactor.

## ► Materials and Methods

### Mice

BALB/c mice (8–12 wks old) were bred and maintained in our laboratory or purchased from Jackson Laboratories (Bar Harbor, ME).

### Abs, cytokines, and other reagents

Anti-Thy-1.2 mAb HO13.4 (29) and anti-Lyt-2.2 mAb AD4.5 (30) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Anti-CD4 (L3T4) mAb RL172.4 (31) was provided by M. J. Bevan (University of Washington, Seattle, WA). Goat anti-mouse isotype-specific Abs were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). LPS from *Escherichia coli* O127:B8 was obtained from Sigma Chemical Co. (St. Louis, MO). CT, CTA, CTB, and goat anti-CTB Ab were obtained from List Biologic Laboratories (Campbell, CA). rCTB was provided by J. Holmgren (Göteborg University, Göteborg, Sweden). Soluble TGF- $\beta$ 1 type IIR, rabbit anti-TGF- $\beta$  Ab, and purified porcine TGF- $\beta$ 1 were obtained from R&D Systems (Minneapolis, MN). Porcine TGF- $\beta$ 1 differs from murine TGF- $\beta$ 1 by a single amino acid (32), but is fully active on mouse cells. TGF- $\beta$ 1 was reconstituted in 4 mM HCl containing 1 mg/ml BSA before use. Human rIL-2 was obtained from Cellular Products (Buffalo, NY).

### B cell preparations and cultures

Mouse spleen cell suspensions were prepared as previously described (5). Cell suspensions were treated with 0.83% ammonium chloride to lyse RBCs. T cells were depleted from cell suspensions by treatment with a mixture of anti-Thy-1.2, anti-Lyt-2.2, and anti-L3T4 mAbs and low-toxicity rabbit complement (Accurate Chemical Co., Westbury, NY) (33). Cells were washed three times with HBSS and resuspended in RPMI 1640 medium supplemented with 10% FBS. B cell preparations contained >90% surface Ig-expressing cells and ~1% residual T cells, as assessed by flow cytometry.

To prepare surface IgA<sup>+</sup> (sIgA<sup>+</sup>) B cells, T cell-depleted spleen B cells ( $2.5 \times 10^8$  cells in 5 ml) were added to 60-mm plastic petri dishes precoated with goat anti-mouse IgA Ab (7  $\mu$ g/ml), and incubated for 70 min at 4°C (33). Flow cytometric analysis showed that the nonadherent B cell population contained <0.1% sIgA<sup>+</sup> cells.

B cells were cultured in 96-well tissue culture plates (flat bottom, 0.32-cm<sup>2</sup> growth area) (Costar, Cambridge, MA) at  $1 \times 10^5$  cells/well in a volume of 160  $\mu$ l in RPMI 1640 medium containing 10% FBS, 50  $\mu$ M 2-ME, 2 mM glutamine, and LPS (12.5  $\mu$ g/ml). Unless indicated, other reagents were added in a volume of 40  $\mu$ l/well 24 h after initiation of the cultures.

### Isotype-specific ELISA

The level of specific isotypes produced in the B cell cultures were determined by ELISA as previously

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described (5). Isotype specificity of the ELISAs was confirmed by assaying a panel of myeloma proteins containing IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM. ELISAs were sensitive to Ab concentrations of 1 to 10 ng/ml.

### Isotype-specific enzyme linked immunospot (ELISPOT) assays

Polystyrene 96-well plates (Millipore, Bedford, MA) containing nitrocellulose inserts were coated with 150  $\mu$ l of affinity-purified goat anti-mouse isotype-specific Ab (1.2  $\mu$ g/ml) in PBS (3). Control wells were incubated with PBS/0.05% Tween-20 (PBST) containing 1% BSA. After overnight incubation, the plates were washed with PBST and blocked with 200  $\mu$ l of 1% BSA/PBST for 1 h at 37°C. Titrated numbers of B cells were added to triplicate wells and incubated for 2.5 h at 37°C. The plates were washed and then incubated with 150  $\mu$ l/well biotin-labeled goat anti-mouse isotype-specific Ab in 1% BSA/PBST. After incubation for 2 h at room temperature, plates were washed and 150  $\mu$ l/well of avidin-peroxidase (Boehringer Mannheim, Indianapolis, IN) at a 1:2000 dilution in PBST was added for 2 h. Spots were developed with 3-amino-9-ethylcarbazole and hydrogen peroxide, and counted using a dissecting microscope. Data are presented as numbers of spot-forming cells per well with backgrounds subtracted.

### Determination of IgA secretion rates

sIgA<sup>+</sup> B cell cultures were stimulated with LPS and 24 h later with CT and CTB in the presence of IL-2. After 5 days in culture, cells were harvested, washed, and the number of IgA secreting cells was analyzed by ELISPOT assay. In parallel, at the end of the 5 day period,  $4 \times 10^5$  cells/well were recultured for 6 h in 96-well plates in a 200- $\mu$ l volume, and IgA levels in the supernatants were determined by ELISA. The IgA secretion rate for the 6-h period was calculated by dividing the amount of IgA secreted during this period by the number of IgA-secreting cells present in the culture as determined by the ELISPOT assay.

### Limiting dilution analysis

sIgA<sup>+</sup> spleen B cells were cultured at various cell densities, ranging from  $10^1$  to  $10^5$ /well, in 96-well tissue culture plates (Costar) in 200  $\mu$ l/well. Forty eight replicate wells were set up for each cell density. Cultures were stimulated for 6 days, and wells were assayed individually for the number of isotype-specific Ig-secreting cells by ELISPOT assay. Wells containing spot-forming cells were scored as positive.

Calculations to determine the frequency of B cell precursors that develop into IgA- or IgM-secreting B cells were based on the Poisson distribution analysis as previously described (3). Briefly, the  $\log_{10}$  of the frequency of negative cultures was plotted against the number of cultured B cells and the precursor frequency was derived graphically from the point at which 37% of cultures were negative. The average number of IgA- or IgM-secreting cells that developed from each precursor (i.e., the clonal burst size) was determined based on the input number of B cells that yielded 37% negative cultures, because positive cultures contain an average of one precursor per culture at this input number. Clonal burst size was calculated using data from 48 wells.

### TGF- $\beta$ bioassay

TGF- $\beta$  activity in culture supernatants was determined by assaying the inhibition of proliferation of mink lung epithelial-like cells (ATCC CCL64) (34). Mink lung epithelial-like cells ( $5 \times 10^4$ /well) were seeded in 96-well flat-bottom microtiter plates in 100  $\mu$ l DMEM containing 2% FBS and incubated overnight. After plates were washed with HBSS, dilutions of test supernatants, or of porcine TGF- $\beta$ 1 as a standard, were added in a final volume of 200  $\mu$ l/well. After a 24-h incubation, [ $^3$ H]-thymidine (2  $\mu$ Ci/well) was added, and then cultures were incubated for an additional 5 h and harvested onto glass fiber filters. Radioactivity was determined using a liquid scintillation counter. Values obtained from test supernatants represent active TGF- $\beta$ , because latent TGF- $\beta$  was not converted to the active form before bioassay. TGF- $\beta$  activity in culture supernatants was confirmed by depleting TGF- $\beta$  from supernatants with rabbit anti-TGF- $\beta$  Ab (5  $\mu$ g/ml) before bioassay.

### Quantitative RT-PCR analysis

Total cellular RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD). TGF- $\beta$ 1 mRNA levels were determined by RT-PCR using standard RNAs under the conditions we have previously described (35). Data are expressed as number of mRNA molecules/ $\mu$ g of total cellular RNA.

## ► Results

### CT and CTB increase IgA secretion by murine spleen B cells

Stimulation of LPS-activated murine total spleen B cells with CT or CTB alone did not significantly increase IgA secretion in the cultures (Table I). However, because CT was reported to increase IgA switching among Peyer's patch B cells and murine B lymphoma cells (26, 27), and because we and others have previously demonstrated that IL-2 can promote IgA secretion by spleen B cells (3, 33), we assessed whether IL-2 affected IgA production by CT- or CTB-stimulated total B cell cultures. As shown in Table I, the addition of combinations of IL-2 with either CT or CTB to total B cell cultures increased IgA secretion by five- to ninefold. In contrast, CT or CTB had little effect on IgM and IgG1 secretion by total spleen B cells, irrespective of whether IL-2 was present (Table I).

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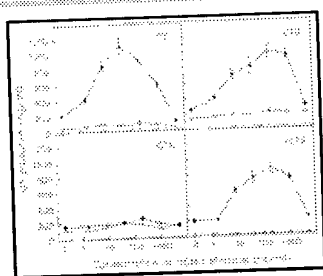
Table 1. *Effect of CT and CTB on isotype production by LPS-stimulated spleen B cells<sup>1</sup>*

To characterize the target cells of CT and CTB, sIgA<sup>+</sup> B cells were prepared and stimulated with either CT or CTB, both with and without IL-2. As shown in Table I, CT and CTB increased IgA secretion in IL-2-stimulated sIgA<sup>+</sup> B cells by 7- and 18-fold, respectively, while they had little effect in the absence of IL-2 costimulation. The relative increase in IgA secretion after CT or CTB stimulation was higher in cultures of sIgA<sup>+</sup> B cells compared with total B cells (Table I), suggesting that the target cells of CT and

CTB were mostly among the sIgA<sup>+</sup> B cells. Furthermore, as in the total B cell cultures, CT or CTB had little effect on the IgM response, but CT and CTB in the presence of IL-2 increased IgG1 secretion by 1.7- to twofold in the sIgA<sup>+</sup> B cell cultures (Table I).

### CT effects on IgA production are mediated by CTB but not CTA

The above studies show that CTB, like CT holotoxin, stimulates IgA secretion. Because purified CTB preparations can contain small amounts of CTA as a contaminant, we determined whether CTA played a role in mediating the increased IgA response to CT holotoxin and CTB. As shown in Figure 1, purified CTA had little or no effect on IgA secretion when tested over a 5000-fold concentration range, irrespective of whether IL-2 was added to the cultures. In contrast, rCTB, which is completely free of CTA, markedly increased the IgA response in a dose-dependent manner when IL-2 was present. Furthermore, rCTB was as efficient as purified CTB and CT holotoxin in inducing maximal IgA responses (Fig. 1), indicating that the CTB component of CT alone is responsible for modulating the IgA response.

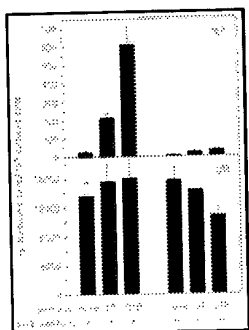


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**FIGURE 1.** CTB but not CTA mediates the CT effect on IgA production. sIgA<sup>+</sup> spleen B cells in 96-well plates were stimulated with LPS (12 µg/ml), and then CT, CTB, CTA, or rCTB at the indicated concentrations were added 24 h later, either without (○) or with (●) 60 IU/ml of IL-2. Supernatants were harvested after 7 days of culture, and IgA levels were assayed by ELISA. Data are means ± SEM of six replicate cultures. Similar results were obtained in two additional experiments, although, as shown in Tables I and III, CTB generally was slightly more effective than CT in increasing IgA responses.

### CT and CTB increase the number of IgA-secreting B cells but not IgA secretion rates

Next, we determined if CT and CTB increased IgA secretion by increasing the number of IgA-secreting cells or, alternatively, by increasing the secretion of IgA-committed B cells already present in culture. sIgA<sup>+</sup> B cells were cultured for 6 days in the absence or presence of CT or CTB, combined with IL-2, and the number of IgA-producing cells was determined by ELISPOT assay. As shown in Figure 2, the addition of CT or CTB to B cell cultures supplemented with IL-2 increased the number of IgA-secreting cells by 6- to 20-fold, an increase similar to that in total IgA secretion (Table I). These data indicate that CT and CTB act mainly to increase the number of IgA-secreting B cells. In contrast to IgA, the number of IgG1- and IgM-secreting cells was not significantly altered in B cell cultures stimulated with either CT or CTB, in the presence of IL-2 (Fig. 2, and data not shown).



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**FIGURE 2.** CT and CTB in combination with IL-2 increase the number of IgA-secreting cells. LPS-stimulated sIgA<sup>+</sup> spleen B cells were cultured for 6 days with CTB (1  $\mu$ g/ml) or CT (0.04  $\mu$ g/ml), with and without IL-2 (60 IU/ml). The number of IgA- and IgM-secreting cells was determined by ELISPOT assay. Data are the means  $\pm$  SEM of six cultures from two separate experiments.

To confirm that CT and CTB did not affect IgA secretion rates in LPS-activated sIgA<sup>+</sup> B cell cultures, the rate of IgA secretion/cell was determined after 5 days of culture by IgA ELISPOT assay and by assays of IgA secretion in short-term culture. Consistent with the above interpretation, calculated amounts of IgA secreted/cell did not increase after CT or CTB stimulation (No stimulation: 678 pg IgA secreted/cell during a 6-h incubation period; CT + IL-2: 307 pg/cell/6 h; CTB + IL-2: 288 pg/cell/6 h; numbers are means,  $n = 2$ ). Furthermore, the spot size in the ELISPOT assays, which we had previously found to correlate positively with the IgA secretion rate/cell (36), did not differ between controls and CT- or CTB-stimulated cells (data not shown).

### CTB increases the IgA precursor frequency

To determine whether CT and CTB, either alone or in the presence of IL-2, increase IgA switching, changes in IgA precursor frequency were determined using limiting dilution analysis (3). As shown in Table II, stimulation of sIgA<sup>+</sup> spleen B cells with CTB and IL-2, but not CTB or IL-2 alone, increased the IgA precursor frequency by more than sixfold. In contrast, CTB and IL-2 did not increase the number of IgA-secreting cells that developed from each IgA precursor (i.e., the clonal burst size). Stimulation with IL-2 alone, as a control, increased the number of IgA-secreting cells per IgA precursor, but did not increase IgA precursor frequency, which is consistent with previously reported data (3). Thus, together with the findings above, these data show that CTB increases IgA switching in the presence of IL-2 but does not increase proliferation and secretion of IgA-committed B cells.

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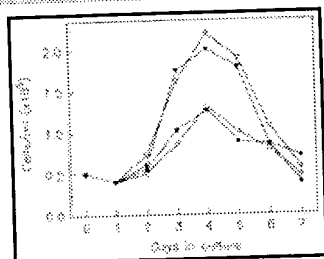
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Table II. Limiting dilution analysis of the IgA precursor frequency in sIgA<sup>+</sup> spleen B cells following CTB stimulation<sup>1</sup>

### CTB inhibits the LPS-stimulated proliferation of sIgA<sup>+</sup> B cells

To further characterize the mechanisms that underlie the CTB-induced increase in IgA secretion, we

determined the effects of CTB and IL-2 stimulation on B cell proliferation. As shown in Figure 3, CTB markedly inhibited the proliferation of LPS-stimulated sIgA<sup>+</sup> B cells, and this inhibition was not affected by the addition of IL-2.



**FIGURE 3.** CTB inhibits B cell proliferation. Cells were cultured in the presence of LPS (▽), LPS + IL-2 (▼), LPS + CTB (○), or LPS + CTB + IL-2 (●). The number of viable cells was determined by trypan blue dye exclusion. Data are means of triplicate culture wells.

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### Increased IgA switching after CTB stimulation is mediated by TGF-β1

The above data show that CTB increases IgA switching and inhibits B cell proliferation. These findings are reminiscent of those previously reported for TGF-β1 (3), which raised the possibility that TGF-β1 mediates the effects of CTB on sIgA<sup>+</sup> B cells. To test this possibility, TGF-β1 activity was neutralized in cultures stimulated with either CT and IL-2 or CTB and IL-2 using an anti-TGF-β Ab or soluble TGF-β1 type IIR. As shown in Table III, anti-TGF-β Ab inhibited the CT- or CTB-stimulated IgA response by >75% in sIgA<sup>+</sup> B cell cultures, while an isotype-matched control Ab had no effect. Anti-TGF-β Ab did not decrease basal IgA secretion in control cultures. Furthermore, addition of soluble TGF-β1 type IIR, which bind active TGF-β1 and prevent it from activating cellular receptors, markedly inhibited the CTB- and IL-2-activated IgA response. These data suggest the CTB-induced increase in IgA secretion is mediated by TGF-β1.

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**Table III.** *TGF-β1 mediates the CTB- and CT-induced increase in IgA production by sIgA<sup>+</sup> spleen B cells<sup>a</sup>*

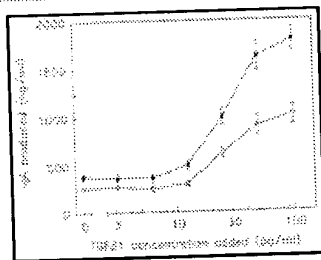
Next, we determined whether TGF-β1 expression and activity was increased in B cell cultures stimulated with CT or CTB, combined with IL-2. First, TGF-β1 mRNA levels were determined by quantitative RT-PCR analysis. CT and CTB in the presence of IL-2 increased TGF-β1 mRNA levels in sIgA<sup>+</sup> B cell cultures by 4.1-fold and 2.7-fold, respectively (Table IV). Next, we assayed the levels of active TGF-β1 after CT and CTB stimulation. Because we previously reported that TGF-β1 is maximally active on IgA switching when added early during the culture period (33), 24-h supernatants from sIgA<sup>+</sup> B cell cultures stimulated with either CT and IL-2, or CTB and IL-2, and control supernatants were assayed for TGF-β activity using the TGF-β sensitive cell line CCL64. In one experiment, supernatants from sIgA<sup>+</sup> B cell



cultures stimulated with either CT and IL-2 or CTB and IL-2 contained 200 pg/ml and 100 pg/ml of active TGF- $\beta$ , respectively, whereas <50 pg/ml of TGF- $\beta$  was detected in IL-2-stimulated or control cultures. In two additional experiments, TGF- $\beta$  levels were below the lower limit of sensitivity of the bioassay (i.e., 50 pg/ml). Nonetheless, these findings are consistent with a role of TGF- $\beta$ 1 in mediating the CT- and CTB-induced IgA response, because levels of active TGF- $\beta$ 1 as low as 25 pg/ml were sufficient to increase IgA secretion by sIgA<sup>+</sup> B cell cultures in the presence of IL-2 (Fig. 4B). The inability to consistently detect active TGF- $\beta$  in culture supernatants could reflect the low total levels produced, with levels sufficient for activation of the IgA response being present in the immediate microenvironment of TGF-producing cells. Additionally, active TGF- $\beta$  could be utilized by the TGF- $\beta$  responsive cells in culture.

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Table IV. *Effect of CT and CTB on TGF- $\beta$ 1 mRNA expression by sIgA<sup>+</sup> B cells<sup>a</sup>*



**FIGURE 4.** TGF- $\beta$ 1 increases IgA production by LPS-stimulated sIgA<sup>+</sup> spleen B cells. The indicated concentrations of TGF- $\beta$ 1 were added to LPS-stimulated sIgA<sup>+</sup> spleen B cell cultures, in the absence (○) or presence (●) of 60 IU/ml IL-2. Supernatants were harvested after 7 days, and IgA levels were assayed by ELISA. Data are the means  $\pm$  SEM of six cultures. Similar results were obtained in two separate experiments.

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## Discussion

The findings herein extend earlier studies on the effect of CT and CTB on murine Ab responses (26, 27, 28). It has been previously shown that CT or CTB increase numbers of IgA-producing cells among populations of LPS-activated Peyer's patch B cells. Also, in the case of CT, activity was shown to target sIgM<sup>+</sup> B cells (26). Further supporting the notion that CT promotes IgA isotype switching, CT increased the precursor frequency of IgA producing-cells in cultures of murine CH12.LX (sIgM<sup>+</sup>) B lymphoma cells (27). In the present study, CTB has been shown to promote IgA isotype switching of LPS-activated normal murine spleen B cells at the clonal level. Moreover, the effects of CTB on the IgA response are shown to be mediated by TGF- $\beta$ 1 and are dependent on IL-2 as a cofactor.

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CTB and IL-2 increased the precursor frequency of IgA secreting-cells within the sIgA<sup>-</sup> B cell population, but they did not increase the burst size of IgA-secreting clones. Thus, CTB and IL-2 did not stimulate the growth of residual B cells in culture that were already committed to the IgA isotype. On the contrary, concentrations of CTB that significantly increased IgA production were shown to inhibit B cell growth. TGF- $\beta$ 1 also markedly inhibits B cell growth (3), but whether the effect on B cell growth was a direct effect of CT and CTB or a secondary effect of TGF- $\beta$ 1 is not known. However, cell cycle inhibitors, including hydroxyurea and thymidine, were shown to increase the number of IgA-secreting cells among populations of LPS-activated sIgA<sup>-</sup> spleen B cells (3), and the ability of CTB to promote IgA switching may be related in part to its inhibitory action on cell growth.

TGF- $\beta$ 1 appeared to be responsible for IgA switching in response to CTB and CT stimulation because the addition of anti-TGF- $\beta$  Ab or soluble TGF- $\beta$ 1 type IIR completely abrogated the increased IgA response induced by CTB and IL-2. This observation was further confirmed by showing that CTB and CT increased TGF- $\beta$ 1 mRNA transcripts in LPS-stimulated sIgA<sup>-</sup> B cell cultures and by the presence of active TGF- $\beta$ , within the range shown to be active on IgA production, in CTB- or CT-stimulated culture supernatants. Taken together, these observations indicate that CTB or CT, in combination with IL-2, promotes IgA isotype switching via TGF- $\beta$ 1. Nonetheless, the source of TGF- $\beta$ 1 is not known. Based on flow cytometric analysis, the sIgA<sup>-</sup> B cell population used in the present study contained <0.1% of sIgA<sup>+</sup> B cells and few, if any, Thy1<sup>+</sup> T cells. Thus, based on the relative numbers of B cells, T cells, and macrophages, and based on the fact that B cells have been reported to produce TGF- $\beta$ 1 (10, 37), sIgA<sup>-</sup> B cells are the likely source of TGF- $\beta$ 1, but we cannot exclude the possibility that residual T cells or macrophages are the source of TGF- $\beta$ 1, or that latent TGF- $\beta$ 1 in the serum is either directly or indirectly activated by CT and CTB.

The CT and CTB effect on IgA switching and production by splenic sIgA<sup>-</sup> B cells required IL-2 as a cofactor, which was not the case in studies by others using Peyer's patch sIgM<sup>+</sup> B cells and sIgM<sup>+</sup> B lymphoma cells (26, 27). Peyer's patch B cells that are induced to the IgA isotype may be at a different developmental stage and have different growth requirements after switching than those in the spleen. Moreover, CH12.LX sIgM<sup>+</sup> B lymphoma cells are known to spontaneously switch at a low frequency to the IgA isotype in the absence of any added stimuli (38). Because IL-2 also increases IgA secretion of B cells induced to undergo IgA isotype switching by TGF- $\beta$ 1 (1, 33), IL-2 may be an essential cofactor for IgA switching because it enables the detection of B cells that have switched to the IgA class by promoting IgA secretion.

Our results have implications for understanding the role of CTB in inducing oral tolerance. This form of tolerance is induced by administering Ag by the enteric or intranasal mucosal route, and has been used to down-regulate systemic immune responses and to treat autoimmune disease in animal models (39). TGF- $\beta$ 1 is one of the major mediators of oral tolerance. Consistent with this observation, enterically administered haptenized colon proteins inhibited 2,4,6-trinitrobenzene sulfonic acid-induced colitis via the induction of TGF- $\beta$ 1, which was likely released from mucosal T cells (40). Proteins coupled to CTB have been shown to induce oral tolerance at doses significantly lower than those required by mucosal administration of the protein alone (24, 41). Based on the studies herein, we suggest that CTB, as a carrier, may prime mucosal lymphoid cells to secrete TGF- $\beta$ 1, and therefore that this is the underlying

mechanism responsible for the CTB effect on oral tolerance. More importantly, in the context of the present study, secreted TGF- $\beta$ 1 may be involved in the concurrent IgA isotype commitment that often accompanies oral tolerance (42, 43, 44).

## ► Footnotes

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<sup>3</sup> Abbreviations used in this paper: CT, cholera toxin; CTB, B subunit of CT; CTA, A subunit of CT; sIgM, surface IgM, sIgA, surface IgA; ELISPOT, enzyme linked immunospot; PBST, PBS/0.05% Tween-20. ■

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